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(54) Title: INTEGRIN-LINKED KINASE, ITS INHIBITORS AND METHODS OF MEDICAL TREATMENT USING THESE INHIBITORS, GENE THERAPY AND PSEUDO-SUBSTRATE INHIBITORS

(57) Abstract

This invention relates to an isolated and purified serine/threonine kinase which is an integrin-linked kinase, designated "ILK". ILK can be used to modulate cell growth, modulate cell adhesion, modulate cell migration and modulate cell invasion. Inhibitors of ILK activity include (1) screens aimed at DNA, RNA or structural components of ILK, (2) pseudo-substrate inhibitors, such as a peptide which mimics a substrate sequence for ILK, and (3) drugs which specifically inhibit ILK activity. The diseases treated by these inhibitors include cancer, leukemia, solid tumors, chronic inflammatory disease, arthritis, osteoporosis and cardiovascular disease. Diagnostics of ILK activity include DNA-based reagents derived from the nucleotide sequence of ILK and antibodies against ILK screen biopsy-derived samples of amplified ILK DNA, or increased expression of ILK mRNA or protein. Assays which screen drugs which specifically inhibit ILK activity are included within this invention.

Integrin-Linked Kinase, Its Inhibitors and Methods of

Medical Treatment Using These Inhibitors, Gene Therapy

and Pseudo-Substrate Inhibitors

5   Background

Proteins of the extracellular matrix (ECM) act to
influence fundamental cell and tissue behaviours. ECM
regulates cell structure, growth, survival,
differentiation, motility and, at the organismal level,
10   proper development.   ECM proteins interact with
cells via a class of cell membrane-spanning receptors
called integrins. ECM is a biological signal, and the
integrin receptor is a specific transducer (i.e. across
the cell's plasma membrane) of this signal. Integrins are
15   also important in proliferative disorders, mediating such
processes as wound healing and inflammation,
angiogenesis, as well as tumour migration and invasion.

A major biochemical response to ECM-integrin
interactions is elevation of an enzymatic activity known
20   as protein phosphorylation. Phosphorylation is important
in signal transduction mediated by receptors for
extracellular biological signals such as growth factors
or hormones. For example, many cancer causing genes
(oncogenes) are protein kinases, enzymes which catalyze
25   protein phosphorylation reactions, or are specifically
regulated by phosphorylation. In addition, a kinase can
have its activity regulated by one or more distinct
protein kinases, resulting in specific signaling
cascades.

Research on signal transduction over the years has clearly established the importance of direct, protein-protein interactions in the cytoplasm as a major mechanism underlying the specification of signaling

5   pathways. These interactions can, in part, be those between a receptor and a cytoplasmic protein kinase, or between a protein kinase and its substrate molecule(s).

A number of known protein kinases, such as mitogen-activated kinase (MAPK), focal adhesion kinase (FAK), and

10 protein kinase C (PKC) have their kinase activity stimulated by integrin-ECM interaction, although no cellular protein kinase has been identified to date, which has been demonstrated to bind to an integrin molecule under physiological conditions. As such is the

15 case, the direct molecular connection between integrins and the ECM-induced phosphorylation of cellular proteins is unclear.

As such is the case, if the direct molecular connection between integrins and the ECM-induced

20 phosphorylation of cellular proteins were determined, products which modulated that connection would be useful therapeutics. These products could be used to modulate cell growth, cell adhesion, cell migration and cell invasion. If it were determined that a specific kinase

25 regulates integrin function, products that regulate (for example, inhibit) the activity of that kinase could be used for the treatment of cancer, leukemia, solid tumors, chronic inflammatory disease, arthritis and osteoporosis, among other indications.

30

Summary of Invention

This invention relates to an isolated and purified serine/threonine kinase which is an integrin-linked kinase, designated "ILK" in this application. ILK binds to the cytoplasmic portion of the β_1 integrin molecule in a living cell, providing the first physiological evidence for interaction between an integrin and a protein kinase.

ILK can be used to modulate cell growth, modulate cell adhesion, modulate cell migration and modulate cell invasion. An amino acid sequence of ILK and an isolated nucleotide molecule encoding the amino acid sequence are part of this invention. The molecule could be cDNA, sense DNA, anti-sense DNA, single DNA, double stranded DNA. mRNA of integrin-linked kinase is part of this invention. The molecule could be a nucleotide molecule encoding an impaired amino acid sequence of a serine/threonine kinase.

Inhibitors of ILK activity are part of this invention. Inhibitors include (1) screens aimed at DNA, RNA or ILK structural components e.g. antisense ILK (i.e. synthetic DNA oligonucleotide comprising the complementary nucleotide sequence of the ILK coding region, designed to specifically target the ILK mRNA complement), (2) pseudo-substrate inhibitors, such as a peptide which mimics a substrate sequence for ILK, and (3) drugs which specifically inhibit ILK activity. These drugs may be directed at either the kinase or ankyrin repeat domains. An inhibitor may include an antibiotic, a natural or mimetic substrate for the integrin-linked kinase, and a first nucleotide molecule which binds to a

second nucleotide molecule of the kinase. The second
nucleotide molecule may be mRNA, cDNA, sense DNA, anti-
sense DNA, single-stranded and double-stranded DNA.

The invention includes a method of treating a
5   disease in a mammal by using an inhibitor of a
serine/threonine kinase, by using a natural or mimetic
substrate for a serine/threonine kinase, or by using a
first nucleotide molecule which binds to a second
nucleotide molecule of the kinase. The method may include
10   gene therapy, for example, the delivery of a gene or cDNA
by any vector (viral or non-viral). The disease may be
one selected from a group consisting of cancer, leukemia,
solid tumors, chronic inflammatory disease, arthritis,
osteoporosis and cardiovascular disease. The carrier for
15   the inhibitor, substrate or molecule would be a
pharmaceutically acceptable carrier, diluent or
excipient. In the case of a nucleotide molecule, a
carrier could be liposomes.

Diagnostics of ILK activity are part of this
20   invention. Diagnostics include nucleotide molecules of
ILK, ILK or its inhibitors. DNA-based reagents derived
from the nucleotide sequence of ILK and antibodies
against ILK screen biopsy-derived samples of amplified
ILK DNA, or increased expression of ILK mRNA or protein.

25   Assays which screen drugs which specifically inhibit
ILK activity are included within this invention. These
assays may be based on the DNA, mRNA or amino acid
sequences of IL-K.

The invention includes a pharmaceutical comprising an inhibitor of ILK activity together with a carrier, for modulating cellular activity.

Figures

5 **FIG. 1** Yeast two-hybrid cloning, characterization, and expression of ILK. **a,** The full length ILK cDNA. **b,** Homology with protein kinase subdomains I to XI. **c,** Amino acid residues comprising ankyrin repeats. **d,** BIT-9 used to probe RNA from human tissues. **e,** Analysis of

10 whole cell lysates of mouse, rat and human cell lines.

FIG. 2 *In vitro* and immune-complex kinase assays. **a,** *In vitro* kinase reactions. **b,** Immune complexes. **c,** ^{32}P-labelled products isolated and analyzed for phosphoamino acid content.

15 **FIG. 3** Antibodies to GST-ILK^{132} recognize p59^{ILK} in integrin co-immunoprecipitations. **a,** Unfractionated polyclonal anti-ILK sera specifically recognize a ^{35}S-methionine, metabolically-labelled cellular protein. **b,** Affinity-purified antibody was adsorbed with GST-ILK

20 agarose-GST. **c,** Polyclonal anti-integrin antibodies used to precipitate surface-biotinylated integrins from PC3 cells,. **d,** Anti-β_1 monoclonal antibodies were used in co-precipitation analyses of lysates of PC3.

FIG. 4 Modulation of ILK kinase activity by ECM

25 components. **a,** ILK phosphorylation of MBP was assayed. **b,** Expression levels of p59^{ILK}. **c,** Representative p59^{ILK} overexpressing clone ILK13-A4a on the ECM substrates. **d,** Adhesion of the ILK overexpressing clones to LN, FN and

VN was quantified. **e**, ILK13, p59^{ILK} overexpressing clones were assayed for colony growth.

FIG. 5 Expression of ILK in human breast carcinomas. **a**, Normal region of breast tissue. **b**, Ductal carcinoma *in situ*. **c,d**, Invasive carcinoma.

Detailed Description of Preferred Embodiments

By this invention, we have shown the physical linkage between integrin and ILK. More importantly, we have shown that dysregulated expression of ILK protein modulates the function of integrins, thus providing a biological link between ILK and integrin. Dysregulated expression of ILK modulates cell growth, cell adhesion, cell migration and cell invasion. Hence, products that inhibit the activity of dysregulated expression of ILK have a therapeutic effect in the treatment of cancer, leukemia, solid tumors, chronic inflammatory disease, arthritis and osteoporosis, among other indications.

The ILK protein is encoded by a 1.8 kilobase pair messenger RNA (1.8 kb mRNA). The sequence of this mRNA was used to deduce the primary amino acid sequence of the protein, which has a predicted molecular weight of 50 kiloDaltons (kDa). The recombinant protein migrates on analytical polyacrylamide electrophoresis gels with an apparent molecular weight of 59 kDa, in rough agreement with the predicted size. The deduced structure of the ILK protein (hereinafter p59ILK) revealed two functional domains, identified by comparison of the ILK sequence against those found in current protein databases. These are the catalytic domain, responsible for phosphotransferase activity (kinase domain), and a non-

overlapping domain in the amino terminus, comprised of four contiguous ankyrin-like.

The function of ankyrin repeats in ILK is to mediate protein-protein interactions. The ILK ankyrin repeat
5   domain is not required for the binding of p59ILK to integrin, and it presumably mediates the interaction of p59ILK with another cellular protein(s). Thus, p59ILK bridges integrin(s) in the plasma membrane, with intracellular proteins active in regulating the cell's
10 response to ECM signals. These proteins are likely to be located in the cytoplasm, or as part of the cell's structural framework (cytoskeleton), but are as yet unidentified.

The novelty of ILK lies in key structural and
15 functional features of the enzyme. Structurally, it represents an unusual molecular architecture, in that a protein kinase and an ankyrin repeat domain are contained within the same protein. The kinase domain is very conserved (i.e. similar) to other kinase sequences in
20 existing databases, and can be divided into typical subdomains (I through XI), based on this conserved structure. However one amino acid in particular is not present in ILK, which is present in a specific context, in subdomain VIb of all other protein kinase domains.
25 Despite this unique structural feature, ILK clearly acts as a protein kinase, and thus could represent a prototype member of a new subfamily of protein kinase molecules.

The commercial potential of ILK is directly linked to its regulation of integrin extracellular activity (ECM
30 interactions) from inside the cell, via its direct

interaction with the integrin subunit (known in the
integrin field as "inside-out" signalling). Interfering
with ILK activity allows the specific targeting of
integrin function, while leaving other essential
5   signalling pathways intact. Moreover, increasing the
levels of cellular ILK activity short circuits the normal
requirement for adhesion to ECM (i.e. integrin function)
in regulating cell growth. Thus, inhibiting ILK activity
would inhibit anchorage-independent (i.e. cancerous) cell
10 growth.

Thus, from a therapeutics point of view, inhibiting
ILK activity has a therapeutic effect on a number of
proliferative disorders, including inflammation and
cancer. Inhibition is achieved in a number of ways: (1)
15 with screens aimed at DNA, RNA of ILK or ILK structural
components e.g. antisense ILK (i.e. synthetic DNA
oligonucleotide comprising the complementary nucleotide
sequence of the ILK coding region, designed to
specifically target the ILK mRNA complement), (2) pseudo-
20 substrate inhibitors, for example, a peptide which mimics
a substrate for ILK, or (3) by assaying inhibition of ILK
activity in an ILK-based functional assay e.g. in vitro
or in vivo ILK kinase activity.

Knowledge of the 3-dimensional structure of ILK,
25 derived from crystallization of purified recombinant ILK
protein, leads to the rational design of small drugs
which specifically inhibit ILK activity. These drugs may
be directed at either the kinase or ankyrin repeat
domains.

From a diagnostics perspective, DNA-based reagents
derived from the sequence of ILK, e.g. PCR primers,
oligonucleotide or cDNA probes, as well as antibodies
against p59ILK, are used to screen biopsy-derived tumours
5   or inflammatory samples e.g. arthritic synovium, for
amplified ILK DNA, or increased expression of ILK mRNA or
protein. DNA-based reagents are designed for evaluation
of chromosomal loci implicated in certain diseases e.g.
for use in loss-of-heterozygosity (LOH) studies, or
10 design of primers based on ILK coding sequence.

Having mapped the ILK chromosomal locus to region
11p15, it was determined that a subset of breast
carcinomas displays LOH for markers in chromosomal region
11p15.5. This region has also been implicated in an
15 inherited form of cardiac arrythmia, the long QT
syndrome. A high level of expression of ILK mRNA
indicates an integrin-independent function for ILK in
cardiac tissue.

Example 1   Isolating cDNA of ILK and ILK

20   A partial cDNA, BIT-9, was isolated in a two-hybrid
screen[4] using a bait plasmid expressing the cytoplasmic
domain of the β_1 integrin subunit. The BIT-9 insert was
used to isolate clones from a human placental cDNA
library. A 1.8 kb clone, Plac5, was found to contain a
25 high degree of similarity to cDNAs encoding protein
kinases (Figure 1 a-c), and recognized a widely expressed
transcript of 1.8 kb in Northern blots (Figure 1 d).
Deduced amino acid residues 186-451 from Plac5 comprise a
domain which is highly homologous with the catalytic
30 domains of a large number of protein tyrosine and

serine/threonine kinases (Figure 1b). Residues 33-164

comprise four repeats of a motif originally identified in

erythrocyte ankyrin[5] (Figure 1 c), likely defining a

domain involved in mediating additional protein-protein

5   interactions.[6, 7]   Affinity-purified anti-ILK antibodies

(see methods described in Example 3) were used in Western

blot analyses of mammalian cell extracts, and detected a

conserved protein of apparent Mr of 59 kDa (p59^{ILK},

Figure 1 e).

10   Figure 1 shows yeast two-hybrid cloning,

characterization, and expression of ILK. **a,** The full

length ILK cDNA, Plac5, was isolated from a human

placental library using the BIT-9 insert. Plac5 contains

a 1509 bp open reading frame, with a presumptive

15   initiator Met[17] at nt 157, and an AAUAAA signal 11 bp

upstream of the polyadenylation site. *In vitro*

transcription and translation of Plac5 in rabbit

reticulocyte lysates yielded a protein of apparent Mr of

59 kDa (not shown). **b,** A search[18] of the PIR protein

20   database indicated homology with protein kinase

subdomains I to XI, as identified by Hanks et al.[19] We

note sequence variations in the ILK subdomains I, VIb,

and VII, relative to catalytic domains of known protein

kinases. Subdomain I (residues 199-213), does not have

25   the typical GXGXXG motif, although this region in ILK is

Gly-rich. In subdomain VIb, Asp^{328} of ILK may compensate

for the lack of the otherwise conserved Asp^{319}. In

subdomain VII, the DFG triplet is absent in ILK. The

integrin binding site maps to amino acid residues 293-451

(BIT-9). The ILK kinase domain is most highly related to the CTR1 kinase of *Arabidopsis thaliana* (30% identity, P < 10^{-13}). The CTR1, B-raf, Yes and Csk kinase domains are aligned with Plac5. c, Amino acid residues 33-164

5   comprise four contiguous ankyrin repeats, as defined by Lux et. al.[5] d, BIT-9 was used to probe a blot of poly A+ selected RNA (MTN I, Clontech) from various human tissues. e, Whole cell lysates of mouse, rat and human cell lines (10 µg/lane) were analyzed by Western blotting

10   with the affinity-purified 92-2 antibody (see description of methods in Example 3). The ILK sequence data are available from GenBank under accession number U40282.

In order to construct integrin 'bait' plasmids[20], sequences encoding amino acid residues 738-798 of the β_1,

15   and residues 1022-1049 of the α_5 integrin subunits were amplified from full-length cDNAs.[21] The primers used were (a) 5' amplification[5'] - GGCCGAATTCGCTGGAATTGTTCTTATTGGC -3' and (b) 3' amplification[5'] - GGCCGGATCCTCATTTTCCCTCATACTTCGG -[3']. PCR products were

20   directionally cloned into pEG202, creating the LexA fusion bait plasmids, pEG202β_1INT and pEG202α_5INT. pEG202β_1INT and pEG202α_5INT repressed β-gal expression from the pJK101 reporter by 50-60% and 70-75%, respectively, in host strain EGY48 (MATα , his3, trp1,

25   ura3-52, LEU2::pLEU2-LexAop6, constructed by Erica Golemis, Massachussetts General Hospital), confirming nuclear expression of the LexA fusions. Co-transformation of baits with the pSH18-34 reporter verified they were transcriptionally inert (not shown). A galactose-

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inducible HeLa cDNA interactor library was present on the

TRP+ vector, pJG4-5 (constructed by Jeno Gyuris, MGH).

For the β_1 interaction trap, EGY48 was transformed

sequentially with pEG202β_1INT, pSH18-34 and pJG4-5, using

5   the lithium acetate protocol[22] (transformation efficiency

= 5-6 x 10^4/μg). 2×10^6 primary transformants were

screened, of which forty-nine interacting clones were

confirmed. The most frequent isolate (31/49) was a 700 bp

insert, BIT-9. Retransformation of EGY48 with the BIT-9,

10   pSH18-34, and pEG202β_1INT plasmids resulted in strong β-

galactosidase expression, confirming the interaction. An

identical screen, using pEG202α_5INT as bait, resulted in

the isolation of 16 positives, none of which were

represented in the set of 49 β_1 interactors. Trapped

15   inserts were used to screen WM35 human melanoma λgt10,

and human placental λgt11 cDNA libraries, using standard

procedures.[23] cDNA sequencing of multiple clones from

each library was done using the dideoxy chain termination

method (Sequenase 2.0, U.S. Biochemical). For data

20   analysis we used the Genetics Computer Group software

package (version 7.0), and database searches were

accomplished via the BLAST[18] server at the National

Center for Biotechnology Information.

Example 2   Analysis of ILK In Vitro

25   For analysis of kinase activity *in vitro*, a

bacterially-expressed fusion protein, GST-ILK[132], was

SDS-PAGE band purified, and incubated with [γ-^{32}P]ATP in

the presence or absence of the exogenous substrate myelin

basic protein (Figure 2). GST-ILK^{132} autophosphorylated

and labelled MBP efficiently in these assays (Figure 2

a). Anti-GST-ILK^{132} (antibody 91-3) immunoprecipitates of

PC3 cell lysates were incubated with [γ-^{32}P]ATP, similar

5   to experiments performed with purified recombinant GST-

ILK^{132}. ILK immune complexes labelled a protein of

apparent Mr of 59kDa (Figure 2 b), corresponding to

p59^{ILK}, as well as cellular proteins of apparent Mr 32

kDa and 70 kDa, which may be endogenous ILK substrates

10   (Figure 2 b). We also see cellular phosphoproteins

(serine/threonine) of approximately 32 kDa and 70 kDa, in

β_1 integrin-specific immune complex kinase assays (not

shown).

In ILK immune complex kinase assays a synthetic

15   peptide representing the β_3 cytoplasmic domain was

phosphorylated, while a similar peptide representing the

β3 cytoplasmic domain was not detectably labelled by

p59^{ILK}. The β_1 peptide selectively inhibited

autophosphorylation of ILK in these reactions (Fig. 2b),

20   further indicating a differential interaction of the

peptides with ILK. The results demonstrating

phosphorylation of synthetic β peptides by endogenous ILK

are identical to those seen with recombinant GST-ILK^{132}

(not shown), and indicate the potential substrate

25   preference of ILK for the β_1 cytoplasmic tail. This does

not, however, necessarily rule out an interaction between

ILK and the β_3 integrin cytoplasmic domain. Phosphoamino

acid analyses, of labelled p59^{ILK} and MBP from the immune

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complex kinase assays detected only phosphoserine in both

substrates (Fig. 2 c), as was the case for

phosphorylation of these substrates by GST-ILK[132] (not

shown). The β_1 peptide was labelled on serine and

5   threonine residues, with approximately equal

stoichiometry (Figure 2). As a control, anti-FAK[8, 9]

immune complexes from the same lysates were analyzed for

phosphorylation of MBP, and phosphotyrosine was readily

detected (not shown).

10   Figure 2 shows *in vitro* and immune-complex kinase

assays. **a,** *In vitro* kinase reactions containing 2 µg of

gel-purified GST-ILK[132] , with and without 5 µg of myelin

basic protein (MBP, Upstate Biotechnologies, Inc.), were

analyzed by 10% SDS-PAGE. **b,** Immune complexes were

15   generated from PC3 whole cell lysates, using affinity-

purified 91-3 antibody. Complexes were assayed for kinase

activity, with and without addition of 5 µg/reaction of

synthetic peptides, representing β_1 or β_3 integrin

cytoplasmic domains,[24] or MBP (not shown). Products were

20   analyzed by 15% SDS-PAGE (kDa markers at left), and

migration of peptides confirmed by Coomassie Blue

staining. **c,** ^{32}P-labelled products from the anti-ILK

immune complex kinase reactions shown in b, were isolated

and analyzed for phosphoamino acid content. Anti-FAK[8, 9]

25   immune complex kinase assays demonstrated phosphotyrosine

on MBP (not shown).

Protein kinase assays were performed in 50 µl kinase

reaction buffer (50 mM HEPES pH 7.0, 10 mM $MnCl_2$, 10 mM

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MgCl$_2$, 2 mM NaF, 1 mM Na$_3$VO$_4$), containing 10 µCi [γ-

^{32}P]ATP. Reactions were incubated at 30°C for 20 min, and

stopped by the addition of SDS-PAGE sample buffer. For

assay of recombinant ILK activity, GST-ILK^{132} was

5   adsorbed from bacterial lysates onto glutathione-agarose

beads, or GST-ILK^{132} was band-purified from 10% SDS-PAGE

gels. For immune complex kinase assays, affinity-purified

91-3 anti-ILK antibody (Fig. 3, Methods) was used to

generate immunoprecipitates from NP-40 lysates (150 mM

10   NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 50

mM HEPES pH 7.5, 1 µg/ml each leupeptin and aprotinin, 50

µg/ml phenyl-methylsulfonyl flouride) of PC3 cells.

Kinase reaction products were resolved on 10-15% SDS-PAGE

gels, transferred to PVDF, and phosphoamino acid analysis

15   performed according to a published protocol.[25]

Example 3   Association of ILK and b integrin In
                   Mammalian Cells

Immunofluorescence experiments indicated that ILK

and β integrin co-localize in focal plaques (not shown).

20   In order to test further for this association in intact

mammalian cells, we performed co-immunoprecipitation

assays in lysates of PC3 cells, in which integrin

expression has been well-characterized.[10] PC3 cell

lysates were immunoprecipitated with specific anti-

25   integrin antibodies,[13] and immune complexes analyzed by

Western blotting with the anti-ILK antibody, 92-2. The

specificities of the anti-ILK antibodies were tested by

immunoprecipitation and Western blotting (Figure 3 a, b).

We detected p59^{ILK} in immune complexes obtained with

anti-fibronectin receptor (FNR, α_5/α_3 β integrin), and

anti-vitronectin receptor (VNR, $\alpha_v\beta_3/\beta_5$ integrin)

antibodies, but not in those obtained with non-immune

5   serum (Figure 3 c). Three anti-β_1 monoclonal antibodies

also co-precipitated p59^{ILK} from PC3 lysates, confirming

the β integrin specificity of p59^{ILK} interaction (Figure

3 d). The detection of p59^{ILK} in anti-VNR immune

complexes suggests that ILK may also interact with the β_3

10   and/or β_5 integrin subunit(s).

Figure 3 shows that antibodies to GST-ILK^{132}

recognize p59^{ILK} in integrin co-immunoprecipitations. **a,**

Unfractionated polyclonal anti-ILK sera 91-3 (shown) and

92-2 specifically recognize a ^{35}S-methionine,

15   metabolically-labelled cellular protein, of apparent Mr

of 59 kDa. A fluorograph is shown (En^{3}Hance, NEN). **b,**

Affinity-purified 92-2 antibody was adsorbed with 165 μg

of agarose-coupled GST-ILK^{132}, or agarose-GST, which

preparations were used in parallel Western blots

20   containing 10 μg/lane of whole cell lysates of PC3 cells,

Jurkat T-lymphoblasts, or the 60 kDa GST-ILK^{132}. **c,**

Polyclonal anti-integrin antibodies, specific for the

fibronectin and vitronectin receptors, were used to

precipitate surface-biotinylated integrins from PC3

25   cells, and immune complexes were then analyzed for the

presence of p59^{ILK}, by Western blotting with affinity-

purified, biotin-labelled 92-2 antibody. This result is

representative of six independent experiments. d, Anti-β_1
monoclonal antibodies were used in co-precipitation
analyses of NP-40 lysates of PC3: lane 1, A$_{II}$B$_2$; lane 2,
anti-CD29; lane 3, 3S3. Western blotting of anti-β_1 immune

5   complexes with affinity-purified, biotinylated 92-2
antibody (left). This blot was stripped and reprobed with
the same concentration of biotinylated 92-2, adsorbed
against an excess of GST-ILK^{132} beads (right). We observe
co-precipitation of p59^{ILK} using a panel of 11 anti-b$_1$

10   monoclonals, but not with an anti-CD44 monoclonal
antibody (not shown). The migration of p59^{ILK} was
confirmed in parallel lanes containing PC3 whole cell NP-
40 lysates. Markers at left, in kDa.

Amino acid residues 132-451 of ILK were expressed as

15   a GST fusion protein, in *E. coli*. Recombinant GST-ILK^{132}
protein was purified and used to inject two rabbits. The
resulting antisera, 91-3 and 92-2 (raised by Research
Genetics, Inc.), were affinity-purified over a column of
CNBr-Sepharose coupled GST-ILK^{132}. PC3 cells were

20   metabolically labelled with 100 μCi/ml [^{35}S]methionine/
[^{35}S]cysteine ([^{35}S] ProMix, 1000 Ci/mmol, Amersham), for
18 hours in cysteine/methionine-free MEM. For co-
immunoprecipitation experiments PC3 cells were surface-
labelled with sulfo-NHS-biotin[26] (Pierce Chemicals),

25   prior to lysis in NP-40 buffer. Polyclonal anti-
fibronectin receptor (anti-FNR, Telios A108), and anti-
vitronectin receptor (anti-VNR, Telios A109) antibodies
were purchased from Gibco/BRL. 1-2 mg of NP-40 lysate was

incubated at 4°C, with 2-3 µl/ml anti-FNR or anti-VNR

antiserum, or 2 µg/ml of the anti-β_1 monoclonal antibodies

A$_{II}$B2 (C. Damsky, UC, San Francisco), anti-CD29 (Upstate

Biotechnology, Inc.), and 3S3 (J. Wilkins, U Manitoba).

5   Lysates were pre-cleared and immune complexes collected

with, Protein A-Sepharose. For Western blotting, RIPA[27]

lysates or immune complexes were subjected to 7.5% or 10%

SDS-PAGE, and proteins then electrophoretically

transferred to polyvinylidene fluoride membranes

10   (Immobilon-P, Millipore). Membranes were blocked in 5%

non-fat milk/Tris-buffered saline Tween-20, and incubated

with 0.5 µg/ml affinity purified antibodies. Horseradish

peroxidase-coupled goat anti-rabbit IgG was used in

secondary incubations, followed by detection of reactive

15   bands by enhanced chemiluminescence (ECL, Amersham). For

blotting without use of secondary antibody (Fig. 3),

affinity-purified 92-2 antibody was labelled with Biotin

Hydrazide (Immunopure, Pierce Chemicals), according to

the manufacturer's protocol, with visualization by

20   peroxidase-conjugated streptavidin (Jackson

ImmunoResearch Laboratories) and ECL. For re-probing,

membranes were stripped according to manufacturer's

instructions.

Example 4   Overexpression of ILK Provides Growth
25   **Advantage**

We next tested for fibronectin-dependent regulation

of ILK kinase activity. Plating of rat intestinal

epithelial cells, IEC-18,[11] on fibronectin reduced ILK

phosphorylation of MBP in immune complex kinase assays,

30   relative to cells plated on plastic, or kept in

- 18 -

suspension (Figure 4 a). This fibronectin-dependent
reduction of ILK activity was abrogated in IEC-18 cells
expressing an activated H-*ras* allele,·[11] indicating that
ras transformation disrupts ECM regulation of ILK
5   activity in these cells. An expression vector containing
the full-length ILK cDNA, pCMV-ILK, was stably
transfected into IEC-18 cells. Twelve stable clones each,
of pCMV-ILK·and vector control transfectants, were
selected and characterized for p59^{ILK} expression levels.
10   Two representative overexpressing subclones, ILK13-A1a3
and -A4a are illustrated (Figure 4b). Overexpression of
p59^{ILK} disrupted the epithelial morphology of IEC-18
cells. ILK13 clones were more refractile, and grew on
LN, FN and VN with a stellate morphology, in marked
15   contrast to the typical, 'cobble-stone' morphology of the
parental and ILK14 cells (Figure 4 c). We plated the
ILK13-A1a3 and -A4a subclones, the control transfectants,
ILK14-A2C3 and -A2C6, and IEC-18 cells, on varying
concentrations of the integrin substrates, laminin (LN),
20   fibronectin (FN) and vitronectin (VN). Adhesion of the
ILK14 and IEC-18 cells was equivalent, whereas that of
the overexpressing subclones was significantly reduced,
on all these substrates (Figure 4 d). Immunoprecipitation
analysis indicated that cell surface integrin expression
25   was unaffected (not shown). The effect of p59^{ILK}
overexpression on anchorage-independent growth was
examined by assaying the colony forming ability of ILK
transfectants in soft agarose. In marked contrast to IEC-
18 and transfectant controls, four independent p59^{ILK}
30   overexpressing subclones, ILK13- A4a, A1a3, A4d3 and

- 19 -

A4C12, formed colonies in these assays (Figure 4 e). The
proliferative rates of all of these clones on tissue
culture plastic were equivalent to control rates.

Figure 4 shows the modulation of ILK kinase activity
5   by ECM components. **a,** ILK phosphorylation of MBP was
assayed in ILK immune complexes, from lysates of IEC-18
intestinal epithelial cells which were harvested from
tissue culture plastic and either kept in suspension, or
replated on fibronectin, for 1 hour. A H-*ras*-transformed
10 variant of IEC-18,[11] Ras37 (transfected with Ras^{val12} in
pRC/CMV vector), was assayed in parallel. The band shown
is MBP. **b,** Expression levels of $p59^{ILK}$ in two
representative clones of IEC-18 cells, transfected with
an ILK expression construct (ILK13), two vector control
15 clones (ILK14), and the parental IEC-18 cells are
presented. The indicated amounts (µg/lane) of whole cell
RIPA lysates were run out on 10% SDS-PAGE gels, and
$p59^{ILK}$ expression analyzed by Western blotting with
affinity-purified 92-2 antibody. **c,** Representative $p59^{ILK}$
20 overexpressing clone ILK13-A4a, vector control clone
ILK14-A2C3, and parental IEC-18 cells were plated on the
ECM substrates LN, FN and VN for 1 hour, then fixed,
stained with toluidine blue and photographed (40x mag).
d, Adhesion of the ILK overexpressing clones to LN, FN
25 and VN was quantified. Key: IEC-18 (black), ILK14-A2C6
(white), ILK13-A1a3 (dark grey), ILK13-A4a (light grey).
Results are presented for 10 µg/ml substrate, and are
expressed as % adhesion (+/- s. d.) relative to IEC-18,
for each substrate. The serial concentrations of ECM
30 showed similar reductions in adhesion of the ILK13

subclones, and ILK14-A2C3 adhesion was identical to that
of ILK14-A2C6, on all three substrates.
Immunoprecipitation of surface-biotinylated IEC-18,
ILK13, and ILK14 subclones, with the anti-FNR and anti-

5   VNR sera, confirmed there was no change in expression of
$\alpha_5/\alpha_3\beta_1$ and $\alpha_v\beta_3/\beta_5$ integrin subunits in the p59^{ILK}
overexpressors (data not shown). Data are representative
of two independent experiments. **e,** Four ILK13, p59^{ILK}
overexpressing clones were plated in soft agarose, and

10   assayed for colony growth after three (experiment 1) and
two (experiment 2) weeks. Parent and vector control
transfectants were also assayed, and the ras^{val12}
transformed clone, Ras-37, was used as a positive
control. Bars represent the mean of duplicate

15   determinations. Maximum colonies in IEC-18 and ILK14
cells was 1/field.

The rat intestinal epithelial cell line IEC-18, and
a variant of this line transfected with an activated H-
ras^{val12} allele, expressed from pRC/CMV, were grown on

20   tissue culture plastic in 5% serum-containing medium,
washed three times in minimum essential medium (MEM), and
harvested with 5 mM EDTA. These were resuspended in 2.5
mg/ml BSA in MEM, and either kept in suspension, or
plated on 10 µg/ml fibronectin-coated plates, for 1 hour

25   at 37°C. NP-40 lysates (300 µg) of these cells were
immunoprecipitated with affinity-purified 91-3, and
immune complex kinase assays (MBP substrate) performed,
as described above. IEC-18 were transfected with the
expression vector pRC/CMV, containing Plac5 in the

30   forward orientation relative to the CMV promotor. Stable

clones were selected in G418, and subcloned through two
rounds of limiting dilution. In all, twelve each of ILK
and vector control transfectant subclones were isolated.
Protein concentrations were determined using the Bradford

5   reagent (Bio-Rad). Two p59^{ILK} overexpressors, ILK13-A1a3
and ILK13-A4a, and two vector transfectant controls,
ILK14-A2C3 and -A2C6, were analyzed for effects of ILK
overexpression on cell adhesion to ECM substrates.

Adhesion was quantified according to published methods.[28]

10   For colony formation assays 3 x 10^5 cells were plated in
35mm wells, in 0.3% agarose, as described previously.[29]
Ras-37 were plated at 2 x 10^3/well. Colonies were counted
and scored per field (d = 1 cm) in duplicate wells, and
defined as a minimum aggregate of 50 cells.

15   These results demonstrate that p59^{ILK} overexpression
in the IEC epithelial cells provides a growth advantage,
in the absence of proliferative signals normally provided
by adhesion.

         The transduction of extracellular matrix signals
20   through integrins influences intracellular ('outside-in')
and extracellular ('inside-out') functions, both of which
appear to require interaction of integrin cytoplasmic
domains with cellular proteins.[12, 13] The association of
ILK with β$_1$ integrin subunits, and specific regulation of
25   its kinase activity by adhesion to fibronectin, suggests
that p59^{ILK} is a mediator of integrin signalling. Thus
the ankyrin repeat motif likely represents a protein
interaction module specifying interactions of ILK with

downstream, cytoplasmic or cytoskeletal proteins. Reduced

ECM adhesion by the p59^{ILK} overexpressing cells is

consistent with our observation of adhesion-dependent

inhibition of ILK activity, and suggests that p59^{ILK}

5   plays a role in inside-out integrin signalling.

Furthermore the p59^{ILK}-induced, anchorage-independent

growth of epithelial cells indicates a role for ILK in

mediating intracellular signal transduction by

integrins.[14-16]

10   **Example 5   The Effect of Anti-ILK On Cell Migration**

The role of ILK in cell motility has important

implications for normal physiological processes such as

inflammation and wound healing, as well as pathological

conditions involving tumour invasiveness and metastatic

15   tumour spread, or osteoporosis (bone is essentially an

extracellular matrix secreted by osteoblast, or bone-

forming cells, and this deposition can be modulated by

integrin expression levels and function). Cell motility

is a dynamic process, which is dependent on integrin-ECM

20   interactions. The "on-off" switch function of protein

kinases provides an ideal mechanism for the dynamic

regulation of integrin affinity states for ECM

substrates. Thus we are currently assaying the effect on

cell migration of microinjecting highly specific anti-ILK

25   antibodies (thereby inhibiting ILK function) into the

cell's cytoplasm. Initially these effects will be assayed

in endothelial cells plated on solid substrata, but will

be extended to include studies on cell migration through

three-dimensional gels composed of ECM proteins.

Example 6   **Anti-S nse Oligonucleotid s to Inhibit**
                   ILK Activity

The sequence of ILK cDNA provides information for
the design and generation of synthetic oligonucleotides
5   for "anti-sense" inhibition of ILK activity. This term
derives from the strategy of employing a reverse
complement of the coding, or sense strand of a specific
messenger RNA, known as an anti-sense oligonucleotide
(AO). By binding to its complementary mRNA, the AO
10   inhibits translation of that mRNA into protein, thereby
preventing normal protein accumulation in the cell. It is
not possible to predict which region of an mRNA will
provide the most efficient translational inhibition,
although we will test ILK AO derived from the ILK mRNA
15   sequence closest to the presumptive translational start
site, as defined in Fig.1, as this provides the most
successful reagents for this.

Regardless of the actual chemistry used to construct
the AO, or modifications to an anti-ILK AO to improve its
20   efficiency, the cDNA sequence of ILK provides the
information for derivation of a specific AO. The cDNA
sequence of ILK is used to design oligonucleotide
reagents, known as degenerate primers (due to the
degeneracy of the genetic code), for use in polymerase
25   chain reaction (PCR)-based screens for cDNAs structurally
related to ILK. Similarly, the ILK cDNA is used to screen
for related genes in a more conventional screen of
genomic or cDNA libraries, by employing less stringent
(i.e. milder) hybridization conditions during screening.
30   In this way, distinct cDNA or DNA sequences significantly
related to ILK (> 50% nucleotide identity) can be

isolated, and a family of ILK-related kinases identified
in a non-random fashion.

Example 7   Mapping of ILK Chromosomal Locus to

Assess Imprinted Copies of Gene

5   We conduct higher resolution mapping of the ILK
chromosomal locus through fluorescent in situ
hybridization (FISH) to metaphase (i.e. separated and
identifiable) human chromosomes has placed the ILK gene
on chromosome 11p15. FISH is known to those skilled in
10   the art. Finer resolution uses known marker genes in this
region. The 11p15 region is indicates that certain genes
(e.g. insulin-like growth factor 2, IGF2) have been shown
to be imprinted (i.e. preferentially expressed from
either the maternally or paternally-derived chromosomes).
15   This imprinting effectively provides a functional
deletion or "knock-out" of one of the two inherited
copies of a gene. Thus mutation of the non-imprinted
allele (copy) has a more profound outcome, since no
compensatory activity is available from the imprinted
20   allele. Also, 11p15 has been identified as a region
subject to loss-of-heterozygosity, or LOH, in a subset of
breast tumour patients. LOH results in the loss of one
allele, for example by gene deletion, and is a mechanism
underlying the contribution of a number of tumour
25   suppressor genes to the development of various cancers
(e.g. BRCA1 in breast, DCC in colon carcinoma, and RB1
in retinoblastoma).

Thus ILK cDNA sequence is used to develop DNA
reagents for the diagnosis and prognostic indications of
30   a significant subset of breast cancers, and these

reagents   contribute to the molecular classification of
such tumours. As mentioned above, the gene(s) on 11p15
contributing to some inherited cases of long QT syndrome
are identified, and the candidacy of ILK as a causative
5 gene for this cardiac condition are evaluated by looking
for alterations in ILK gene structure, in families where
11p15 associations have been made.

Example 8   **Induction of *in vivo* Tumorigenesis by**

10   **Overexpression of ILK**

        Overexpression of ILK down-regulates E-cadherin
which is an important epithelial cell adhesion molecule
mediating cell-cell interactions (Dedhar et al.,
unpublished observations). The loss of E-cadherin
15 induced by overexpression of ILK in epithelial cells
suggests that ILK may promote tumorigenicity *in vivo*. To
test this, we injected cells expressing varying levels of
ILK into athymic nude mice subcutaneously. Mice were
inoculated subcutaneously with the cells expressing high
20 (ILK13-Ala3 and A4a) or low (IEC-18 and ILK14-A2C3)
levels of ILK (10^7 cells/mouse in PBS). The mice were
monitored for tumor formation at the site of inoculation
after three weeks. Tumors arose within three weeks in
50% to 100% of the mice injected with the ILK13 cells (10^7
25 cells/mouse) that overexpress ILK, whereas no tumors were
detected in the mice that were injected with the same
number of the IEC-18 or ILK14 cells expressing lower
levels of ILK (Table I). Thus, overexpression of ILK in
these epithelial cells promotes tumor formation in vivo.

TABLE I: Tumorigenicity of ILK Overexpressing IEC-18 Cells

Cell Line	Number of Mice with Tumors at 3 weeks
IEC-18	0/6
ILK14-A2C3	0/6
ILK13-A1a3	6/6
ILK13-A4a	3/6

Example 9   Increased expression of ILK in human breast carcinoma

5

The expression of Integrin Linked Kinase in human breast carcinomas was determined by immunohistochemical straining of paraffin embedded sections from human breast cancer biopsies.

10   Affinity purified anti-ILK polyclonal antibody was used followed by conjugated secondary antibody. The positive staining observed was completely abolished by absorption of the antibody to ILK-coupled sepharose beads. The photomicrographs represent

15   sections from two tumor samples. A total of 30 samples have been examined so far. In every case ILK expression levels are markedly elevated in tumor tissue compared to normal ducts and lobules. Figure 5A shows a normal region showing well formed ducts

20   with a single layer of epithelial cells. ILK staining is most prominent in epithelial cells. The stroma appears negative. Figure 5B shows ductal carcinoma *in situ* (DCIS). Multiple cell layers are present with markedly elevated ILK staining in the

25   tumor cells. Invasive carcinoma is depicted in

- 27 -

figures 5C and 5D. There is markedly elevated
expression of ILK compared to the normal tissue
shown in figure 5A.

5 _____

The present invention has been described in terms of
particular embodiments found or proposed by the present
inventor to comprise preferred modes for the practice of
10   the invention. It will be appreciated by those of skill
in the art that, in light of the present disclosure,
numerous modifications and changes can be made in the
particular embodiments exemplified without departing from
the intended scope of the invention. For example, due to
15   codon redundancy, changes can be made in the underlying
DNA sequence without affecting the protein sequence.
Moreover, due to biological functional equivalency
considerations, changes can be made in protein structure
without affecting the biological action in kind or
20   amount. All such modifications are intended to be
included within the scope of the appended claims.

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We Claim:

1. An isolated and purified serine/threonine kinase
    which is an integrin-linked kinase.

2. The serine/threonine kinase of claim 1 for a use
    selected from a group consisting of modulating cell
    growth, modulating cell adhesion, modulating cell
    migration and modulating cell invasion.

3. An isolated nucleotide molecule of the kinase of
    claim 1.

4. The molecule of claim 3 which is selected from a
    group consisting of mRNA, cDNA, sense DNA, anti-sense
    DNA, single-stranded DNA and double stranded DNA.

5. An isolated nucleotide molecule encoding an impaired
    amino acid sequence of a serine/threonine kinase
    which is an integrin-linked kinase.

6. An inhibitor of the serine/threonine kinase of claim
    1.

7. The inhibitor of claim 6, wherein the inhibitor is an
    antibody.

8. The inhibitor of claim 6, wherein the inhibitor is a
    a natural or mimetic substrate for the integrin-
    linked kinase.

9. The inhibitor of claim 6, wherein the inhibitor is a
    first nucleotide molecule which binds to a second
    nucleotide molecule of the kinase.

10. The inhibitor of claim 8, wherein the second
    nucleotide molecule is selected from a group

consisting of mRNA, cDNA, sense DNA, anti-sense DNA,
single-stranded DNA and double stranded DNA.

11. A method of treating a disease in a mammal by using
an inhibitor of a serine/threonine kinase.

12. A method of treating a disease in a mammal by using a
natural or mimetic substrate for a serine/threonine
kinase.

13. A method of treating a disease in a mammal by using a
first nucleotide molecule which binds to a second
nucleotide molecule of the kinase.

14. The method of claim 13, wherein the second nucleotide
molecule is selected from a group consisting of mRNA,
cDNA, sense DNA, anti-sense DNA, single-stranded DNA
and double stranded DNA.

15. The method of claim 10, claim 11, claim 12 or claim
13 wherein the method modulates cellular activity and
wherein the cellular activity is selected from a
group consisting of cell growth, cell adhesion, cell
migration and cell invasion.

16. The method of claim 10, further comprising
administering to the mammal a pharmaceutical
comprising the inhibitor and a carrier.

17. The method of claim 10, claim 11, claim 12 or claim
13 wherein the disease is one selected from a group
consisting of cancer, leukemia, solid tumors, chronic
inflammatory disease, arthritis, osteoporosis and
cardiovascular disease.

18. A pharmaceutical comprising an inhibitor of the
serine/threonine kinase of claim 1 together with a

carrier, for modulating cellular activity, wherein
the cellular activity is selected from a group
consisting of cell growth, cell adhesion, cell
migration and cell invasion.

5   19. The pharmaceutical of claim 18, wherein the inhibitor
is a natural or mimetic substrate for the
serine/threonine kinase.

20. A pharmaceutical comprising a first nucleotide
molecule which binds to a second nucleotide molecule
10   of the serine/threonine kinase of claim 1 together
with a carrier, for modulating cellular activity,
wherein the cellular activity is selected from a
group consisting of cell growth, cell adhesion, cell
migration and cell invasion.

15 21. A diagnostic kit comprising the nucleotide molecule
of claim 3 or claim 4.

22. A diagnostic kit comprising the serine/threonine
kinase of claim 1.

23. A diagnostic kit comprising the inhibitor of claim 6.

20 24. An assay for screening inhibitors of a
serine/threonine kinase of claim 1 comprising the
kinase of claim 1.

25. An assay for screening inhibitors of a
serine/threonine kinase comprising the nucleotide
25   molecule of claim 3 or claim 4.

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```
gaattcatctgtcgactgctactaccacgggaggagttccccggagaaggatcctgcagcccgagt      60
ccgaggataaagcttgggggttcatcctcccctgatcctccacagtcctcaggct                 120
tccccaatccaggggactcggcgccggagacgctgctatggacgacatttcactcagtgc            180
                                            M  D  D  I  F  T  Q  C        8

CGGGAGGGCAACGCAGTCGCCGTTCGCCTGTGGCTGGACAACACGGAGAACGACCTCAAC            240
 R  E  G  N  A  V  A  V  R  L  W  L  D  N  T  E  N  D  L  N               28

CAGGGGGACGATCATGGCTTCTCCCCTTGCACTGGGCCGGAGAGGGCCGCTCTGCT                300
 Q  G  D  D  H  G  F  S  P  L  H  W  A  C  R  E  G  R  S  A               48

GTGGTTGAGATGTTGATCATGCGGGGGCACGGATCAATGTAATGAACCGTGGGGATGAC            360
 V  V  E  M  L  I  M  R  G  A  R  I  N  V  M  N  R  G  D  D               68

ACCCCCCTGCATCTGGCCAGCCAGTCATGGACACCGTGATATTGTACAGAAGCTATTGCAG          420
 T  P  L  H  L  A  A  S  H  G  H  R  D  I  V  Q  K  L  L  Q               88

TACAAGGCAGACATCAATGCAGTGAATGAACACGGGAATGTGCCCTGCCACTATGCCTGT          480
 Y  K  A  D  I  N  A  V  N  E  H  G  N  V  P  L  H  Y  A  C              108

TTTTGGGGCCAAGATCAAGTGGCCAGAGGACCTGGTGGCCAAATGGGCCCTTGTCAGCATC         540
 F  W  G  Q  D  Q  V  A  E  D  L  V  A  N  G  A  L  V  S  I             128

TGTAACAAGTATGGAGAGATGCCTGTGGACAAAGGCCACCCCTGAGAGAGCTTCTC              600
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Fig. 1a

SUBSTITUTE SHEET (RULE 26)

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C  N  K  Y  G  E  M  P  V  D  K  A  K  A  P  L  R  E  L  L     148
CGAGAGCGGGCAGAGAAGATGGGCCAGAATCTCAACCGTATTCCATACAAGGACACATTC   660
R  E  R  A  E  K  M  G  Q  N  L  N  R  I  P  Y  K  D  T  F     168
TGGAAGGGGACCACCCGGCCACTCGGCCCCGAAATGGAACCCTGAACAAACACTCTGGCATT  720
W  K  G  T  T  R  P  R  N  G  T  L  N  K  H  S  G  I           188
GACTTCAAACAGCTTAACTTCCTGACGAAGCTCAACGAGAATCACTCTGGAGAGCTATGG   780
D  F  K  Q  L  N  F  L  T  K  L  N  E  N  H  S  G  E  L  W     208
AAGGGCCGCTGGCAGGGCAATGACATTGTCGTGAAGGTGCTGAAGGTTCGAGACTGGAGT   840
K  G  R  W  Q  G  N  D  I  V  V  K  V  L  K  V  R  D  W  S     228
ACAAGGAAGAGCAGGGACTTCAATGAAGAGTGTCCCGGCTCCAGGATTTTCTCGCATCCA   900
T  R  K  S  R  D  F  N  E  E  C  P  R  L  R  I  F  S  H  P     248
AATGTGCTCCCAGTGCTAGGTGCCTGCCAGTCTCCACCTGCTCCTCCTACTCTCATC     960
N  V  L  P  V  L  G  A  C  Q  S  P  P  A  P  H  P  T  L  I     268
ACACACTGGATGCCGTATGGATCCCTCTACAATGTACTACACGAAGGCACCAATTTCGTC  1020
T  H  W  M  P  Y  G  S  L  Y  N  L  H  E  G  T  N  F  V        288
GTGGACCAGAGCCAGGCTGTGAAGTTTGCTTTGGACATGGCAAGGGGCATGGCCTTCCTA  1080
V  D  Q  S  Q  A  V  K  F  A  L  D  M  A  R  G  M  A  F  L     308
CACACACTAGAGCCCCTCATCCCACGACATGCACTCAATAGCCGTAGTGTAATGATTGAT  1140
H  T  L  E  P  L  I  P  R  H  A  L  N  S  R  S  V  M  I  D     328
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Fig. 1a (continued on page 3/23)

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GAGGACATGACTGCCCGAATTAGCATGGCTGATGTCAAGTTCTCTTTCCAATGTCCTGGT   1200
 E   D   M   T   A   R   I   S   M   A   D   V   K   F   S   F   Q   C   P   G     348
CGCATGTATGCACCTGCCTGGGTAGCCCCGAAGCTCTGCAGAAGAAGCCTGAAGACACA   1260
 R   M   Y   A   P   A   W   V   A   P   E   A   L   Q   K   K   P   E   D   T     368
AACAGACGCTCAGCAGACATGTGGAGTTTGCAGTGCTTCTGTGGGAACTGGTGACACGG   1320
 N   R   S   A   D   M   W   S   F   A   V   L   L   W   E   L   V   T   R       388
GAGGTACCCTTTGCTGACCTCTCCAATATGGAGATTGGAATGAAGGTGGCATTGGAAGGC   1380
 E   V   P   F   A   D   L   S   N   M   E   I   G   M   K   V   A   L   E   G     408
CTTCGTACCATCCCACCAGGTATTCCCTCATGTGTGTAAGCTCATGAAGATCTGCATG   1440
 L   R   T   I   P   P   G   I   S   P   H   V   C   K   L   M   K   I   C   M     428
AATGAAGACCCTGCAAAGCGACCCAAATTTGACATGATTGTGCCTATCCTTGAGAAGATG   1500
 N   E   D   P   A   K   R   P   K   F   D   M   I   V   P   I   L   E   K   M     448
CAGGACAAGtaggactggaaggtcctgcctgaactccagaggtcggggacatggttgg   1560
 Q   D   K   *                                                                   451
gggaatgcacctccccaaagcagcaggcctctggttgcctcccccgcctccagtcatggt   1620
actaccccagcctggggtccatcccctcccccctaccactgtgcgcaagaggggc   1680
gggctcagagctttgtcacttgccacatggtgtctcccaacatgggaggatcagccccg   1740
cctgtcacaataaagtttattatgaaaaaaaaaaaaaaaaaaaa   1786
```

Fig. 1a

```
                     I                    II

Csk    .NMKELKLLQ  TIGKGEFGDV  MLGDYRGN.K  VAVKCIKNDA  .TAQ.....AF
Yes    IPRESLRLEV  KLGQGCFGEV  WMGTWNGTTK  VAIKTLKPGT  MMPEAFLQ..
Ctrl   IPWCDLNIKE  KIGAGSFGTV  HRAEWHGS.D  VAVKILMEQD  FHAE.RVNEF
B-raf  IPDGQITVGQ  RIGSGSFGTV  YKGKWHG..D  VAVKMLNVTA  PTPQQ.LQAF
Ilk    IDFKQLNFLT  KLNENHSGEL  WKGRWQGN.D  IVVKVLDKVR  DWSTRKSRDF   235


         III          IV                    V

Csk    LAEASVMTQ  LRHSNLVQLL  GVIVEE.KGG  LYIVTEYMAK  GSLVDYLRSR
Yes    .EAQIMKK   LRHDKLVPLY  AVVSEE...P  IYIVTEFMTK  GSLLDFLKEG
Ctrl   LREVAIMKR  LRHPNIVLFM  GAVTQPP..N  LSIVTEYLSR  GSLYRLLHKS
B-raf  KNEVGVLRK  TRHVNILLFM  GYSTKP...Q  LAIVTQWCEG  SSLYHLHII
Ilk    NEECPRLRI  FSHPNVLPVL  GACQSPPAPH  PTLITHWMPY  GSLYNVLHE.   283


                       VIa                    VIb

Csk    GRSV.LGGDC  LLKFSLDVCE  AMEYLEGN..  .NFVHRDLAA  RNVLVS.E
Yes    EGKF.LKLPQ  LVDMAAQIAD  GMAYIERM..  .NYIHRDLRA  ANILVG.D
Ctrl   GAREQLDERR  RLSMAYDVAK  GMNYLH.NRN  PPIVHRDLKS  PNLLV.DK
B-raf  ETKFEMI..K  LIDIARQTAQ  GMDYLHAK..  .SIIHRDLKS  NNIFLH.E
Ilk    GTNFVVDQSQ  AVKFALDMAR  GMAFLH.TLE  PLIPRHALNS  RSVMI.DE    329
```

Fig. 1b (continued on page 5/23)

```
                VII                                          VIII
Csk    DNVAKVSDFG  LTK.....EA  SSTQDTGKLP  VKWTAPEALR  ...EKKFSTK
Yes    NLVCKIADFG  LARLIED.NE  YTARQGAKFP  IKWTAPEAAL  ...YGRFTIK
Ctrl   KYTVKVCDFG  LSRLKAS.TF  LSSKSAAGTP  .EWMAPEVLR  ...DEPSNEK
B-raf  DLTVKIGDFG  LATVKSRWSG  SHQFEQLSGS  ILWMAPEVIR  MQDKNPYSFQ    372
Ilk    DMTARIS...  MADVKFSFQC  PGRM.YA..P  .AWVAPEALQ  KKPEDTNRSS

                IX                                           X
Csk    SDVWSFGILL  WEIYSFGRVP  YPRIPLKD.V  VPRVEKGY..  KMDAPDGCPP
Yes    SDVWSFGILL  TELVTKGRVP  YPGMVNRE.V  LEQVERGY..  RMPCPQGCPE
Ctrl   SDVYSFGVIL  WELAT.LQQP  WGNL.NPAQV  VAAVGFKCK.  RLEIPRNLNP
B-raf  SDVYAFGIVL  YELMT.GQLP  YSNINNRDQI  IFMVGRGYLS  PDLSKVRSNC    418
Ilk    ADMWSFAVLL  WELVTR.EVP  FADLSNMEIG  MK.VALEGL.  R.TIPPGISP

                XI
Csk    AVYEVMKN   CWHLDAAMRP  SFLQLREQLE  HIKTHEL
Yes    SLHELMKL   CWKKDPDERP  TFEYIQSFLE  .......
Ctrl   QVAAIIEG   CWTNEPWKRP  SFATIMDLLR  PL.....
B-raf  PKAMKRLMAECLKKKRDERP  LFPQILASIE  LLARSLP                     451
Ilk    HVCKLMKI   CMNEDPAKRP  KFDMIVPILE  KMQDK..
```

Fig. 1b

ANKYRIN

CONSENSUS   -G-TPLH-AA--GH---V--LL--GA--N----
                                 A   D

ANK1   [33]HGFSPLHWACREGRSAVVEMLIMRGARINVMNR
ANK2   GDDTPLHLAASHGHRDIVQKLLQYKADINAVNE
ANK3   HGNVPLHYACFWGQDQVAEDLVANGALVSICNK
ANK4   YGEMPVDKAKAPLRELLRERAEKMGQNLNRIPY[164]

Fig. 1c

Fig. 1d

Fig. 1e

Fig. 2a

SUBSTITUTE SHEET (RULE 26)
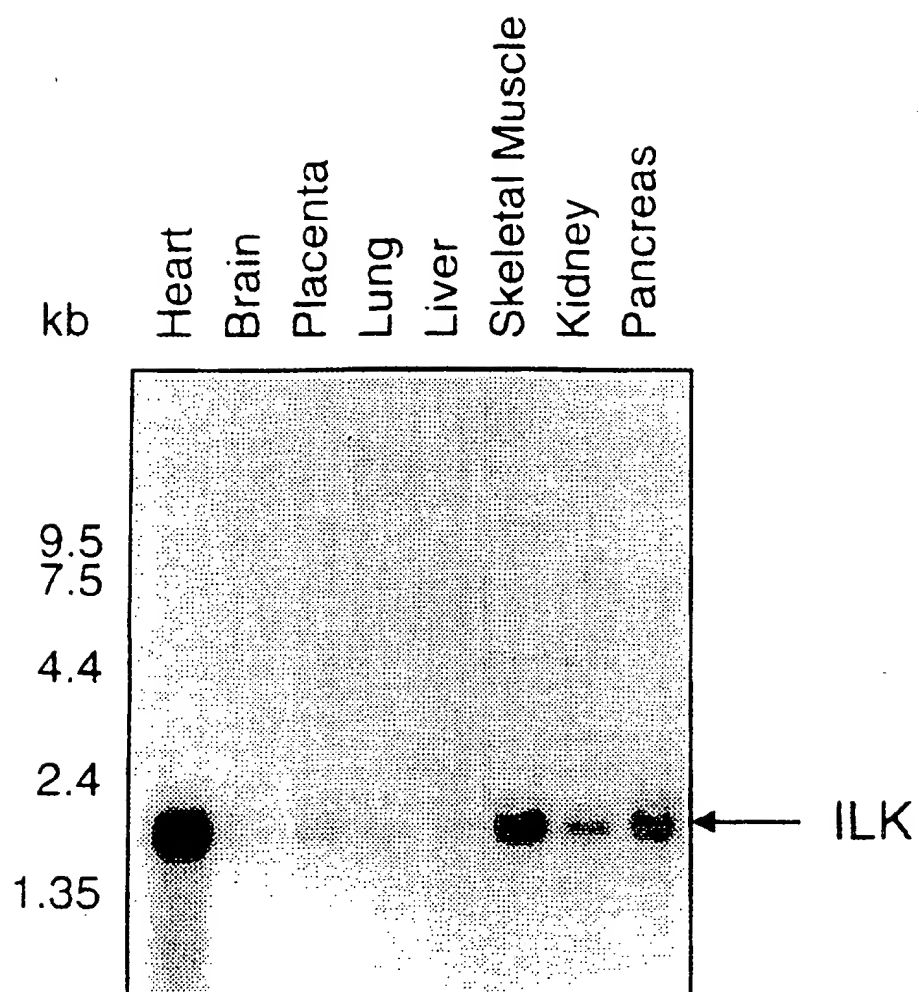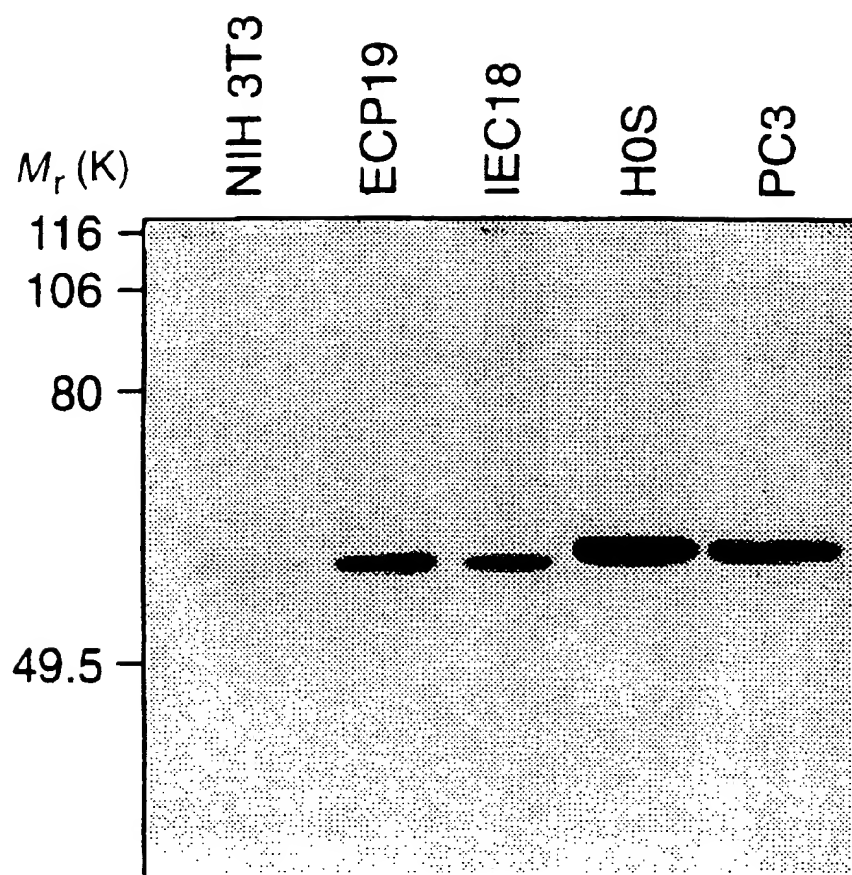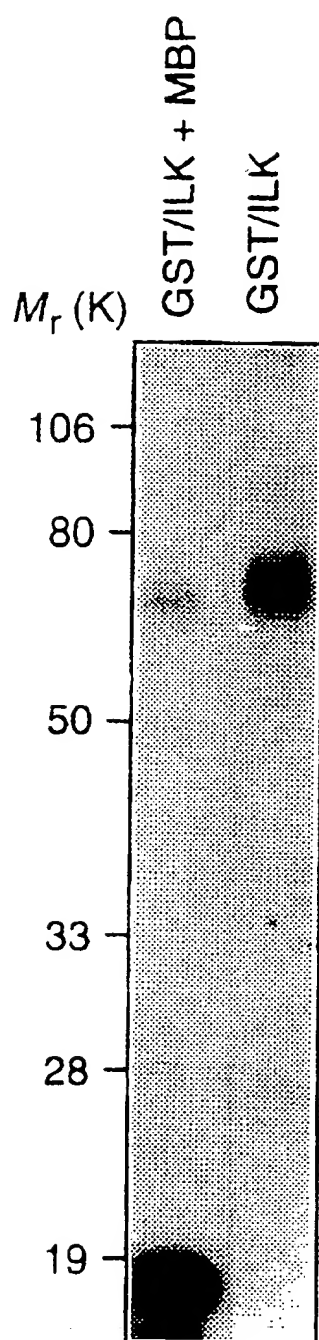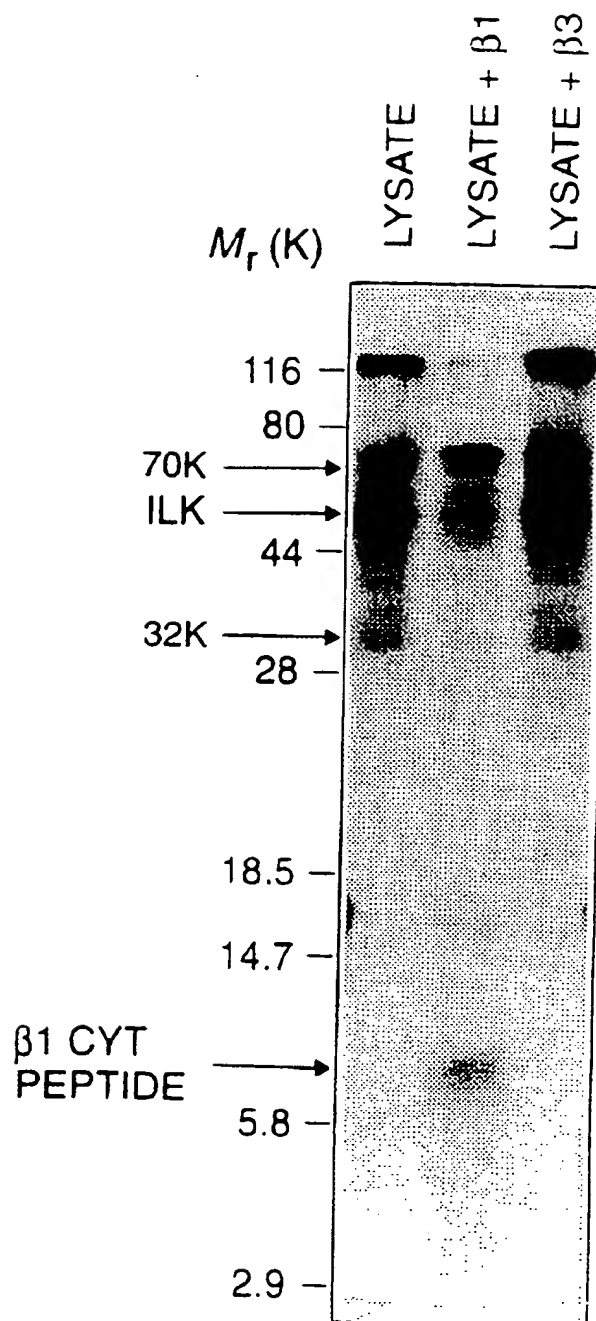
Fig. 2b
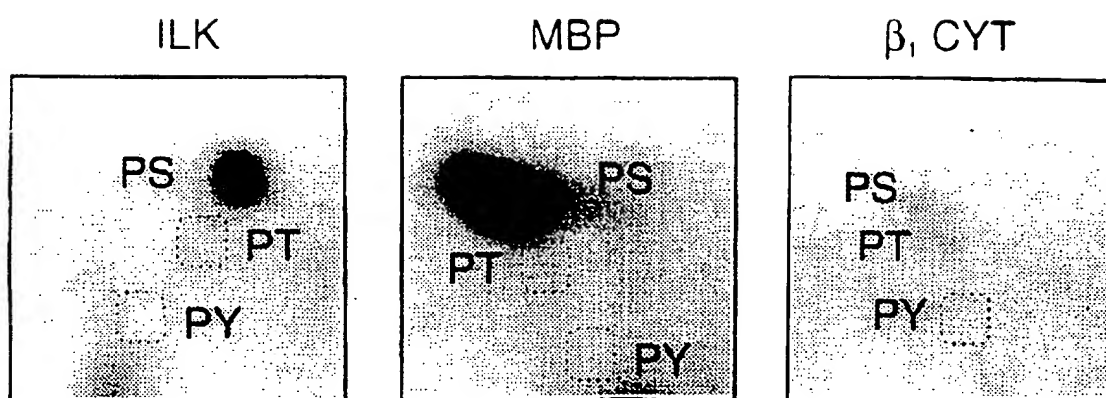
Fig. 2c
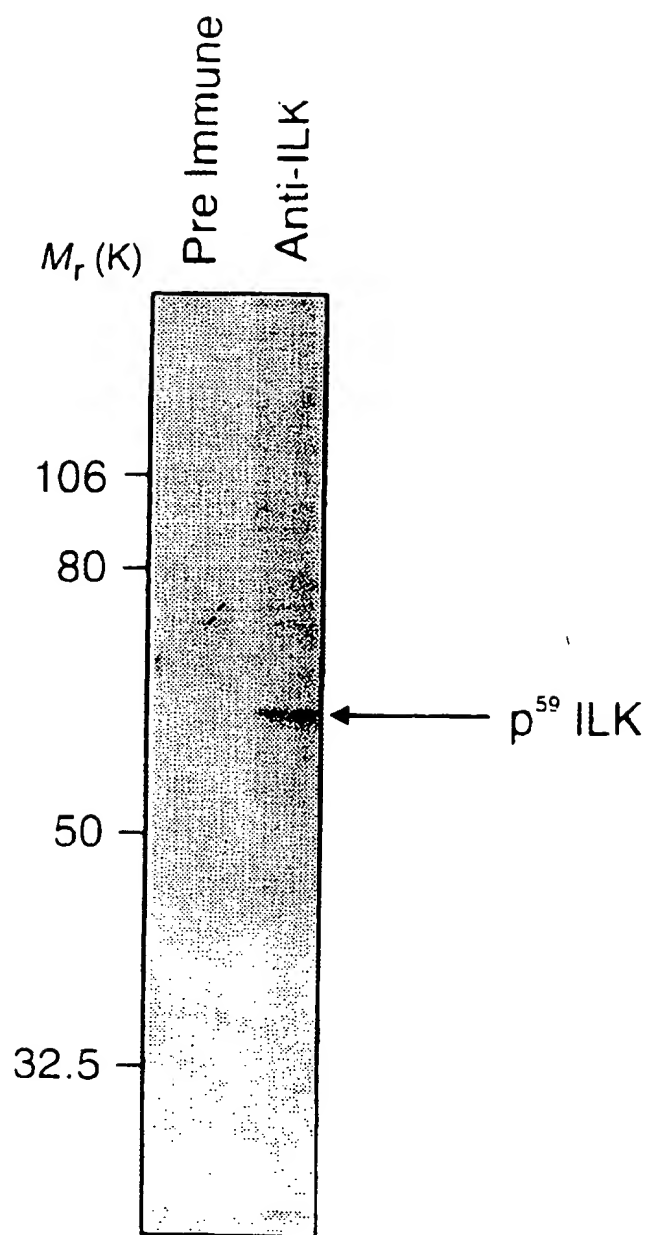
Fig. 3a
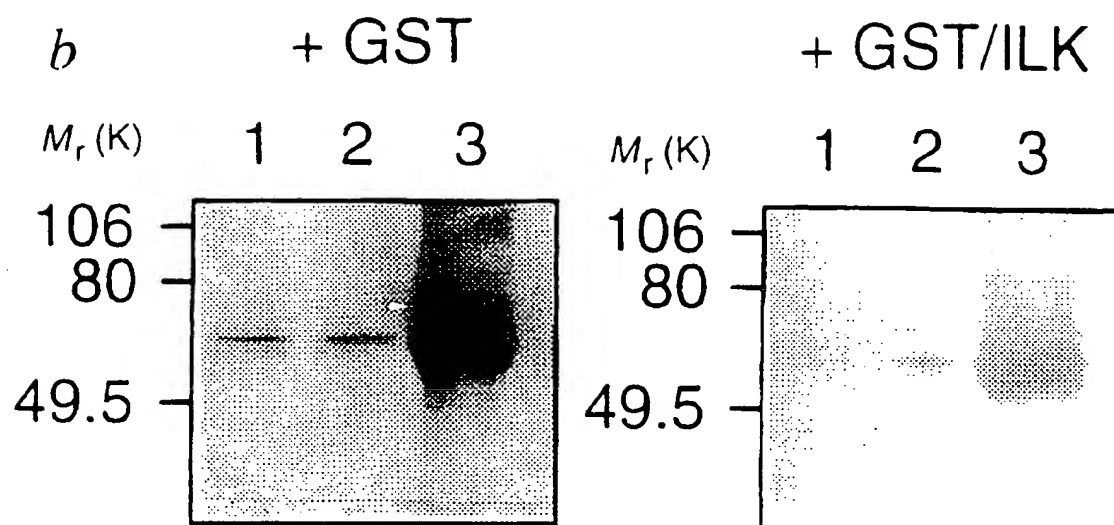
Fig. 3b
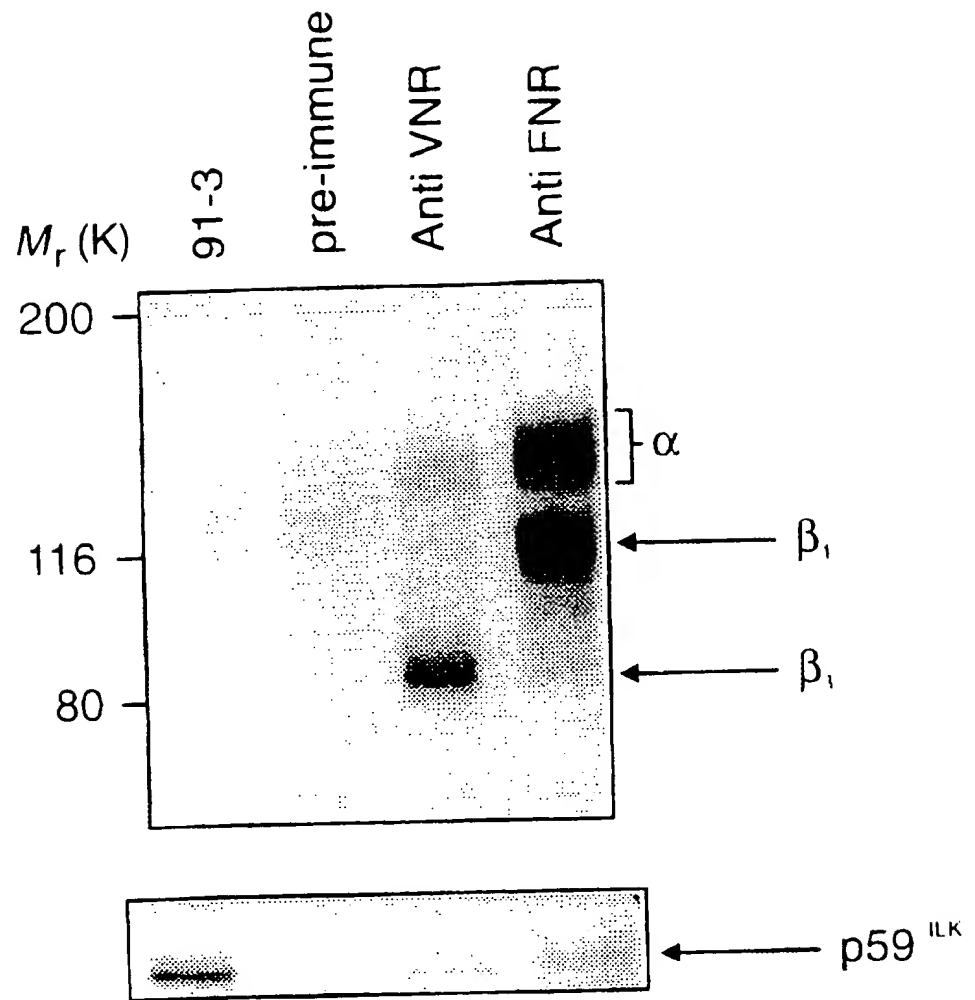
Fig. 3c

Immunoprecipitation: anti β1 monoclonal antibodies

Immunoblot: anti-ILK　　　　adsorbed anti-ILK
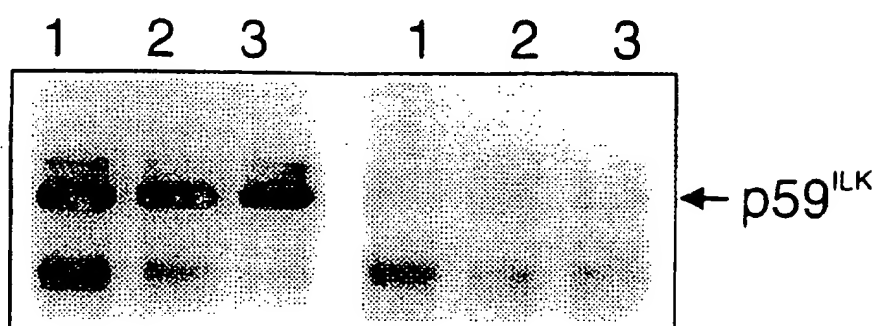
Fig. 3d
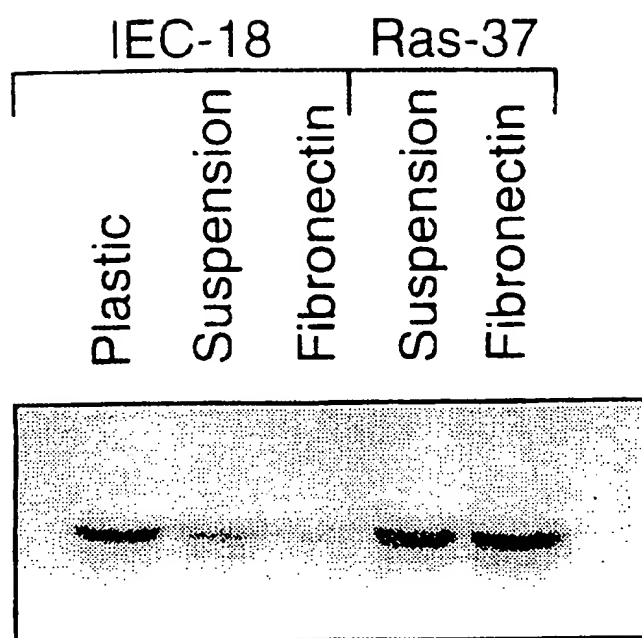
Fig. 4a
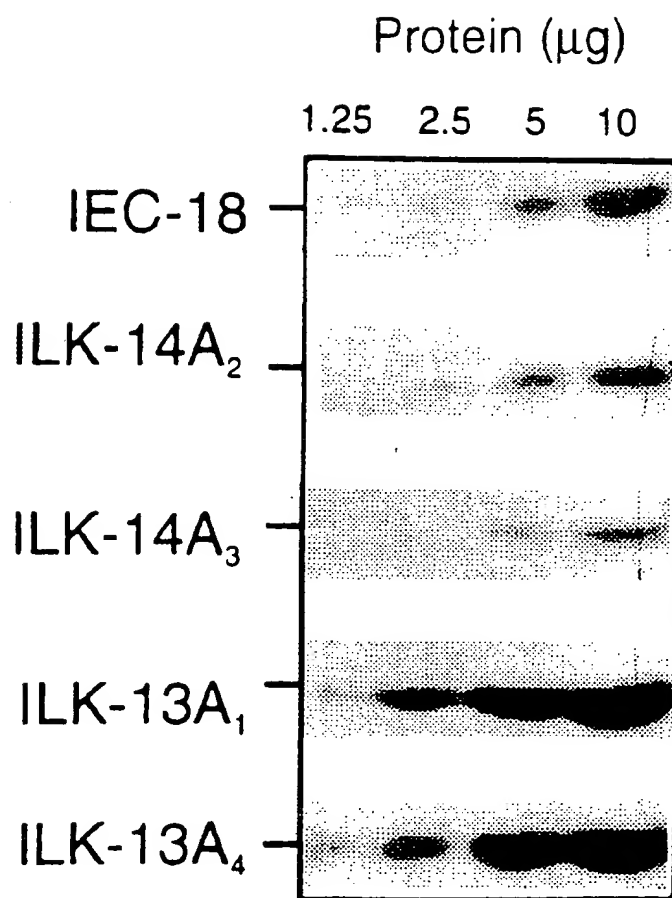
Fig. 4b
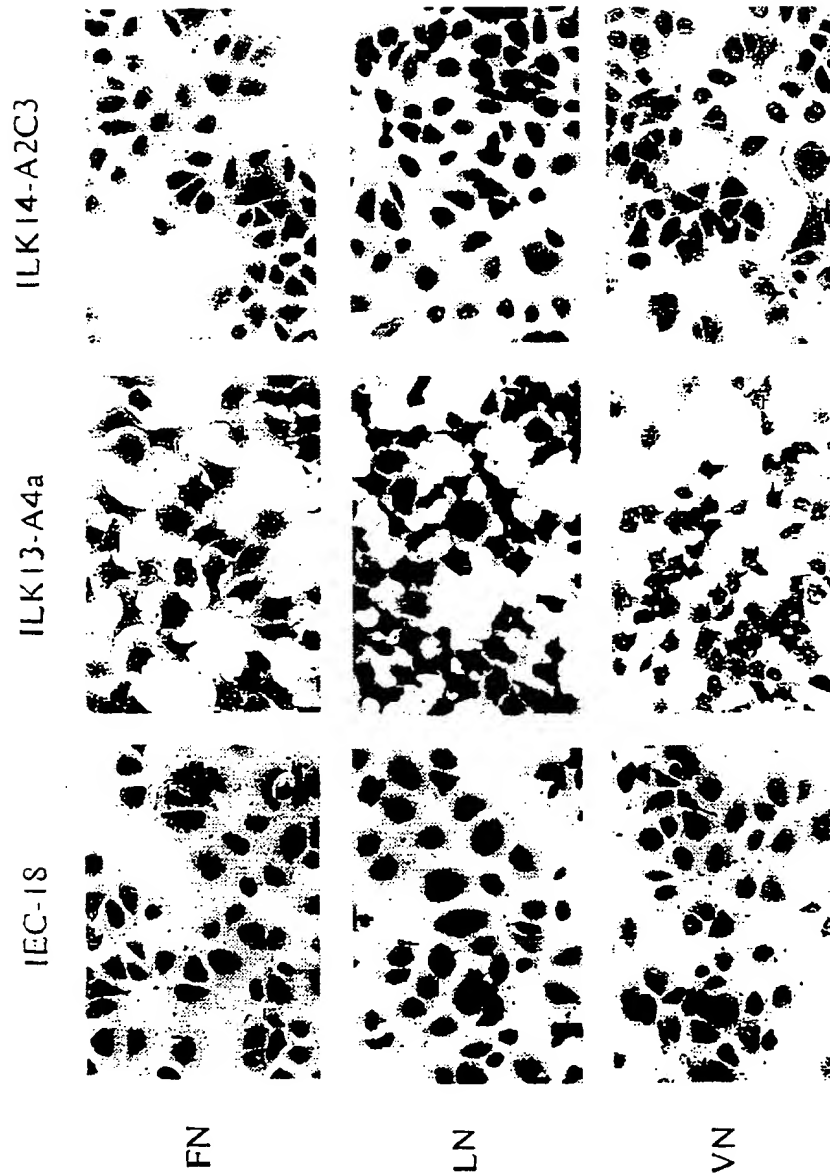
Fig. 4c
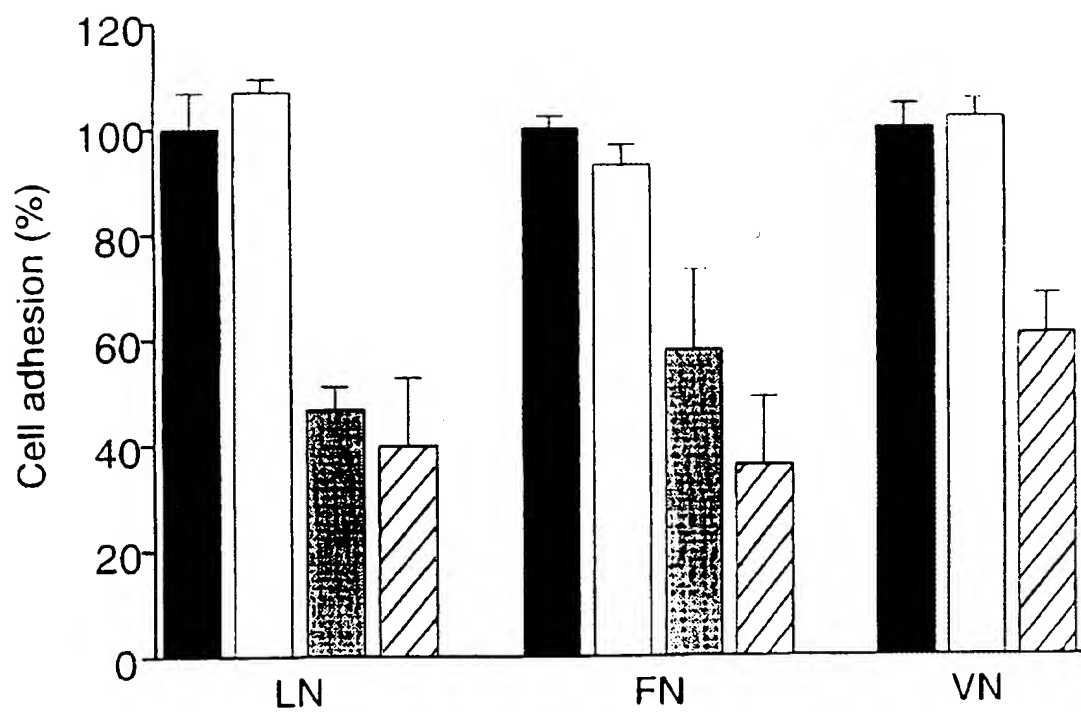
Fig. 4d
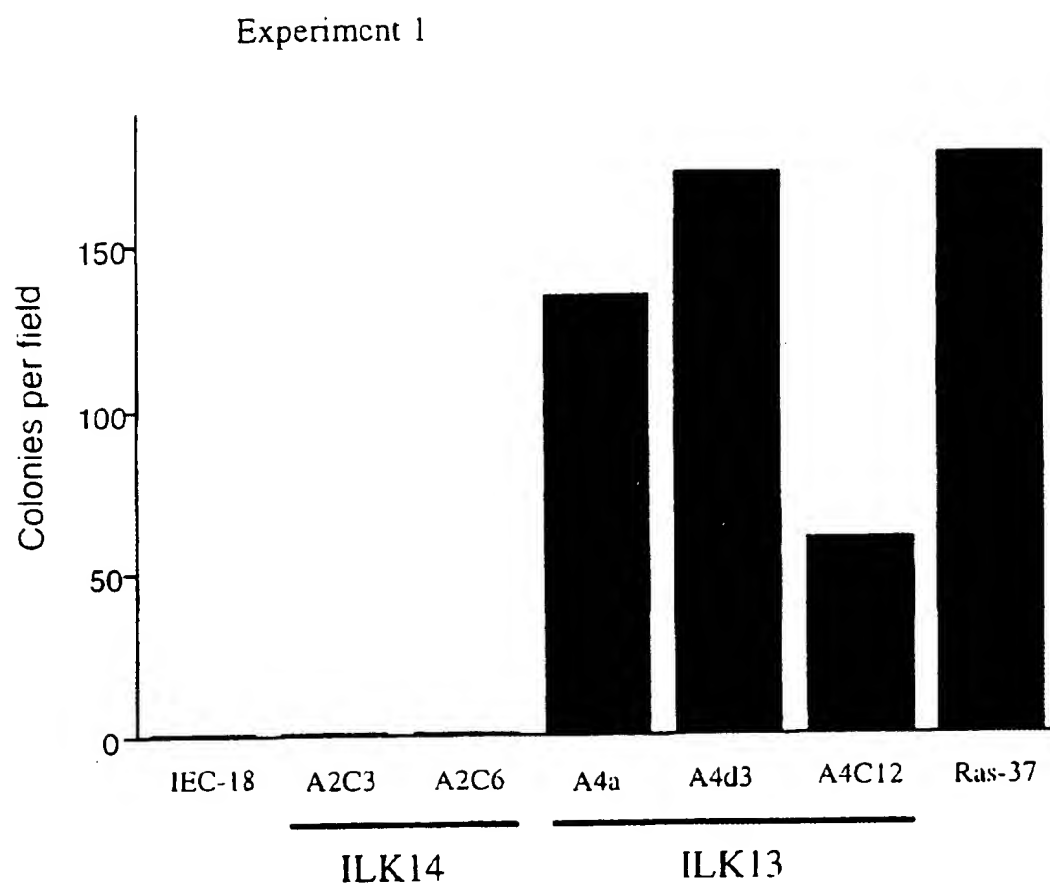
Experiment 1

Fig. 4e (continued on page 21/23)
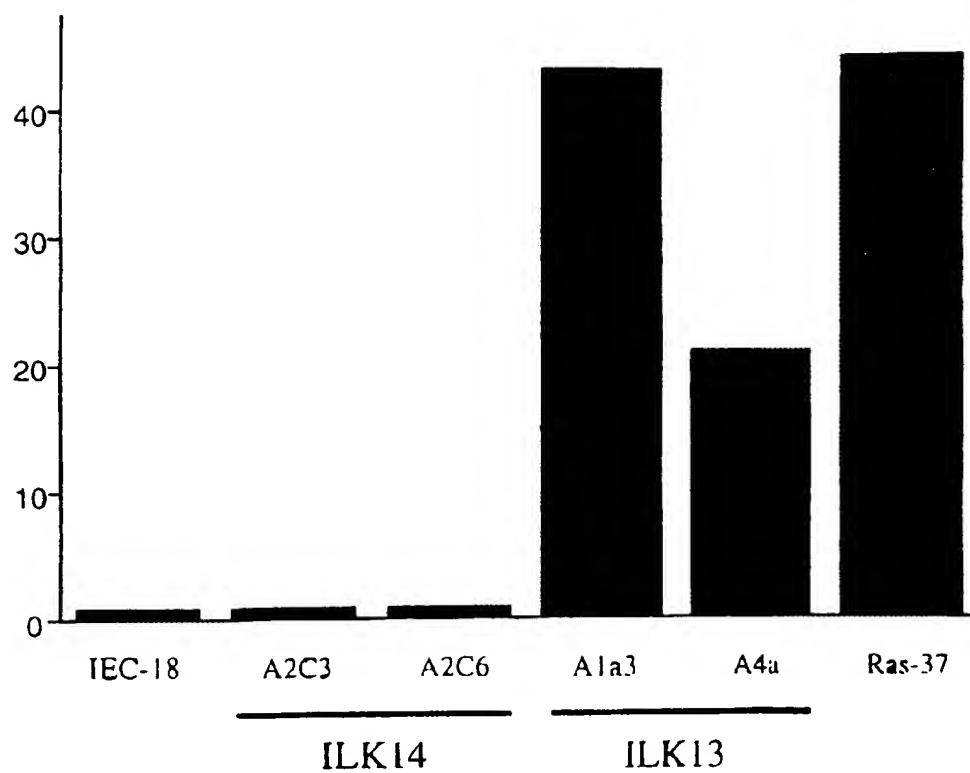
Fig. 4e
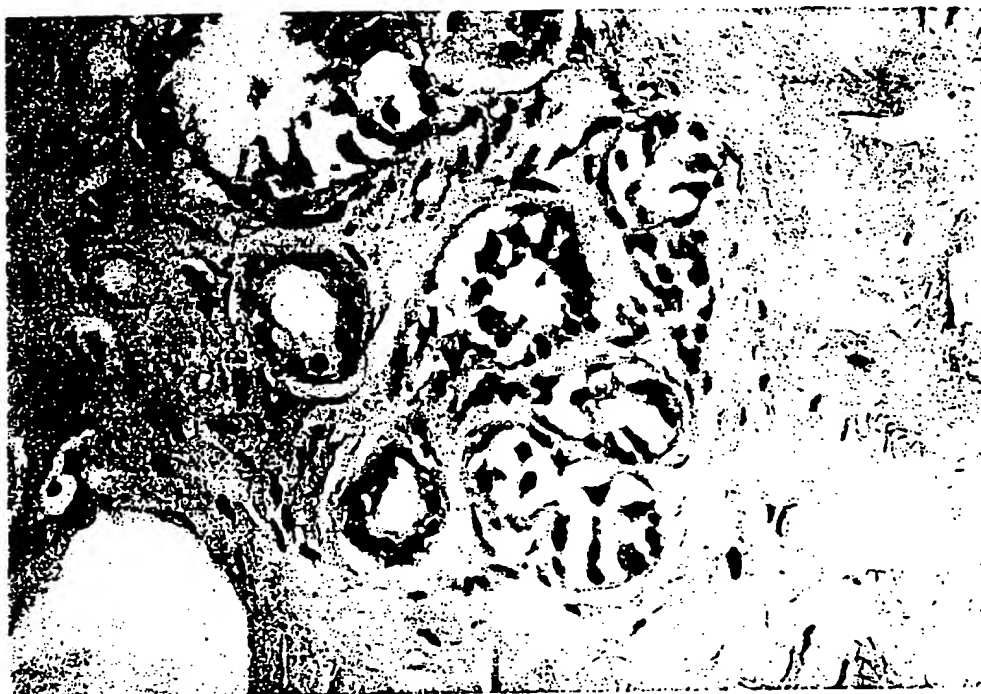
Fig. 5a

Fig. 5b

Fig. 5c

Fig. 5d

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/52   C12N9/12   A61K38/48   A61K31/70   G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C07K   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	86TH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, March 1995, TORONTO, ONTARIO, CANADA, page 361 XP000673939 HANNIGAN, G.E., ET AL.: "CLONING OF A NOVEL PROTEIN KINASE ASSOCIATED WITH BETA1-INTEGRIN CYTOPLASMIC TAILS" see the whole document ---	1-4
X	MOLECULAR BIOLOGY OF THE CELL, vol. 6, no. SUPPL., November 1995, page 2244 XP000673935 HANNIGAN,G.E., ET AL.: "OVEREXPRESSION OF A NOVEL INTEGRIN LINKED KINASE (ILK) INDUCES A TRANSFORMED PHENOTYPE AND CYCLIN D1 EXPRESSION" see the whole document --- -/--	1-4

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

Date of the actual completion of the international search	Date of mailing of the international search report
28 May 1997	0 3. 06. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Holtorf, S

1

International Application No

PCT/CA 96/00760

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 1, 6 January 1995, pages 269-273, XP002031634 MORINO, N., ETAL . : "MATRIX/INTEGRIN INTERACTION ACTIVATES THE MITOGEN-ACTIVATED PROTEIN KINASE, p44erk-1 AND p42erk-2" see the whole document ---	1,2
X	EMBL SEQUENCE DATA LIBRARY, 25 October 1995, HEIDELBERG, GERMANY, XP002031635 HILLIER, L. , ET AL.: "THE WASHU-MERCK EST PROJECT" ACCESSION No. H70160 ---	3,4
X	WO 94 21781 A (MAX PLANCK GESELLSCHAFT) 29 September 1994 see the whole document ---	11,15
X	EP 0 616 032 A (MITSUBISHI CHEM IND) 21 September 1994 see the whole document ---	13-15
P,X	CURRENT OPINION IN CELL BIOLOGY, vol. 8, no. 5, October 1996, pages 657-669, XP000673950 DEDHAR,S. AND HANNIGAN,G.E.: "INTEGRIN CYTOPLASMIC INTERACTIONS AND BIDIRECTIONAL TRANSMEMBRANE SIGNALLING" see the whole document ---	1
P,X	NATURE, vol. 379, 4 January 1996, pages 91-96, XP002031636 HANNIGAN, G.E., ET AL . : "REGULATION OF CELL ADHESION AND ANCHORAGE-DEPENDENT GROWTH BY A NEW BETA1-INTEGRIN-LINKED PROTEIN KINASE" see the whole document ---	1-4
E	WO 96 36642 A (DAVIS ROGER J ;GUPTA SHASHI (US); RAINGEAUD JOEL (FR); DERIJARD BE) 21 November 1996 see the whole document -----	13-15,17

1

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   11-17
   because they relate to subject matter not required to be searched by this Authority, namely:
   Remark: Although claims 11-17 (as far as in vivo methods are concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/ composition.

2. [X] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
   Remark: Claim 16 was read as referring to claim 11; the claims 15 and 17 accordingly referring to the preceding claims 11,12,13 and 14.

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                               [ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9421781 A	29-09-94	US	5459036 A	17-10-95
		AU	6511994 A	11-10-94
		CA	2158544 A	29-09-94
		EP	0689588 A	03-01-96
		JP	8510897 T	19-11-96
EP 0616032 A	21-09-94	JP	6253835 A	13-09-94
		JP	6329551 A	29-11-94
		CA	2116460 A	03-09-94
WO 9636642 A	21-11-96	AU	4904696 A	29-11-96